Isolation of an iron-binding protein from cow's milk

During an investigation of the chromatographic preparation of lactoperoxidase and "red protein" from cow's milk whey, a colorless protein was isolated. In its physical properties and chemical composition it resembles very closely the red protein described by GROVES[1]. On the addition of inorganic iron to the protein, a colored product, with spectral characteristics and iron-binding capacity quite similar to those of the red protein, is formed. These observations suggest that cow's milk contains uncombined, iron-binding protein as well as red protein and that GROVES' method for isolating the latter might be improved by the addition of iron before the process of isolation is begun; such addition has already been applied to studies of the red protein in human milk by SCHÄFER *et al.*[2] and JOHANSON[3].

The iron-binding protein was isolated as follows: The method of MORRISON *et al.*[4] was used for the adsorption of lactoperoxidase, iron-binding protein, and red protein from rennet whey by means of Amberlite IRC-50 (finer than 200 mesh), for the elution of adsorbed proteins by their batch procedure, and for the precipitation of eluted protein by ammonium sulfate. The precipitated protein was dialyzed, lyophilized, and then fractionated by the method of TISELIUS *et al.*[5] on columns of calcium phosphate prepared according to ANACKER AND STOY[6]. Chromatography was carried out, first on a 46 × 420-mm column of hydroxylapatite equilibrated with 0.1 *M* sodium phosphate buffer (pH 6.8). About 2 g of protein dissolved in 50 ml of the buffer were added to the column and chromatography was begun with 0.2 *M* phosphate (pH 6.8), at a flow rate of 60 ml/h. One hundred 10-ml fractions of effluent were combined as a first fraction which contained little protein. The buffer concentration was changed to 0.25 *M* (pH 6.8), and a green solution, also about 1 l in volume, which contained about half of the total protein and most of the lactoperoxidase activity originally applied, was collected as a second fraction. Finally, 0.6 *M* phosphate (pH 6.8) was used to elute the remaining protein as a third fraction, 1 l in volume and faint red in color. The third fraction was dialyzed, lyophilized and fractionated again in the same way. This time the first 1500 ml of effluent contained only a trace of protein. From the next 1000 ml about 70% of the weight of the protein applied was recovered as a practically colorless product and from a final 500-ml fraction, the remaining 30% as a reddish protein. It is estimated that 1–2 g of colorless protein can be prepared in this manner from 100 l of skimmed milk.

The colorless protein is essentially homogeneous in the Tiselius apparatus with a mobility of −0.37 (veronal buffer pH 8.4, *I* 0.1, concn. 1%) and also in the ultracentrifuge with a sedimentation coefficient of 5.1 S (phosphate buffer pH 7.0, *I* 0.1, concn. 1%). GROVES[1] reported values of −0.22 and +5.3 S, respectively, for similar measurements on the red protein.

The iron-binding capacity of the colorless protein was determined by adding 0.1-ml portions of a solution of ferrous ammonium sulfate (10 μg Fe per 0.1 ml) to 26.8 mg protein dissolved in 2.5 ml of the veronal buffer–citrate–carbonate mixture (pH 7.5), described by WARNER AND WEBER[7]. The solution was allowed to stand 10 min after each addition and its absorption at 465 mμ was read in a 1-cm cuvette in a Beckman* spectrophotometer against a buffer blank containing equal amounts

* It is not implied the U.S. Department of Agriculture recommends the above company or its product to the exclusion of others in the same business.

of iron. The absorbancy readings, corrected for changes in volume, are plotted in Fig. 1. The intersection of the two straight lines at 33 μg Fe may be taken as a measure of the iron-binding capacity of the protein on the assumption that the absorption of the original protein solution at 465 mμ is not due to iron. Because of the limited supply of the "iron-free" protein, direct estimations of its iron content were not made. It is perhaps fortuitous that the iron-binding capacity as determined from Fig. 1 is 0.12 % Fe, exactly the same as GROVES' value for the iron content of the red protein.

The red protein prepared in the preceding experiment was examined in a recording spectrophotometer. Its absorption spectrum is very similar to that of GROVES' red protein (ref. 1, Fig. 6). The absorptivity at the 465-mμ maximum is 0.46 (1-cm light path, 1 % solution, pH 7.5) whereas GROVES found 0.49 for his Preparation C. However, the absorptivity at 280 mμ, 12.0, is considerably lower than the 15.1 found by GROVES.

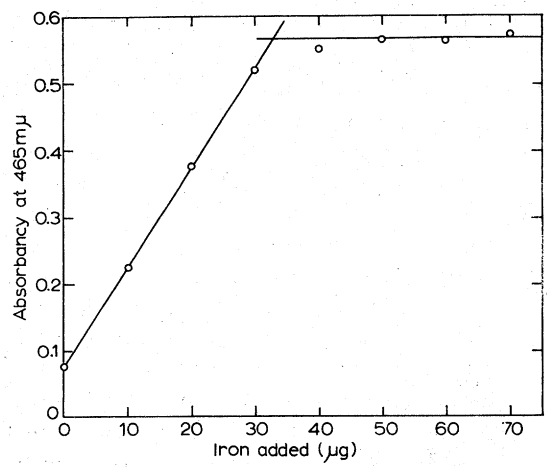

Fig. 1. Iron-binding capacity of colorless milk protein as determined by addition of ferrous iron to 26.8 mg protein dissolved in 2.5 ml buffer mixture; absorbancies read in a 1-cm cuvette have been corrected for changes in volume.

The hexose content of the protein, 4.4 % as determined by the orcinol method described by WINZLER[8], is approximately the same as that reported by GROVES. Two amino acid analyses made with automatic recording equipment agree very well with analyses of GROVES' red protein and provide further evidence that the two proteins are very closely related, if not identical. Results of a detailed study of the amino acid composition of GROVES' preparations are to be published elsewhere.

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